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**TRANSMITTAL  
FORM**

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission

Application Number

10/657,814

Filing Date

8 September 2003

First Named Inventor

Yong-Ha Park

Art Unit

1651

Examiner Name

Attorney Docket Number

1768-041-03

**ENCLOSURES (check all that apply)**☐ Fee Transmittal Form☐ Fee Attached☐ Amendment / Reply☐ After Final☐ Affidavits/declaration(s)☐ Extension of Time Request☐ Express Abandonment Request☐ Information Disclosure Statement☒ Certified Copy of Priority Document(s)☐ Reply to Missing Parts/  
Incomplete Application☐ Reply to Missing Parts  
under 37 CFR1.52 or 1.53☐ Drawing(s)☐ Licensing-related Papers☐ Petition☐ Petition to Convert to a  
Provisional Application☐ Power of Attorney, Revocation  
Change of Correspondence Address☐ Terminal Disclaimer☐ Request for Refund☐ CD, Number of CD(s) \_\_\_\_\_☐ Landscape Table on CD☐ After Allowance Communication to TC☐ Appeal Communication to Board  
of Appeals and Interferences☐ Appeal Communication to TC  
(Appeal Notice, Brief, Reply Brief)☐ Proprietary Information☐ Status Letter☒ Other Enclosure(s)  
(please identify below):  
return postcard**Remarks**

Cert copy of priority doc #10-2001-0011797

Cert. copy of priority doc #PCT/KR2001/002310

**SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT**

Firm

Graybeal Jackson Haley, LLP

Signature

Printed Name

John M. Janeway

Date

23 May 2005

Reg.  
No.

45,796

**CERTIFICATE OF TRANSMISSION/MAILING**

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date shown below.

Signature

Typed or printed name

Laura J. Jordan

Date

23 May 2005

This collection of information is required by 37 CFR 1.5. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.



별첨 사본은 아래 출원의 원본과 동일함을 증명함.

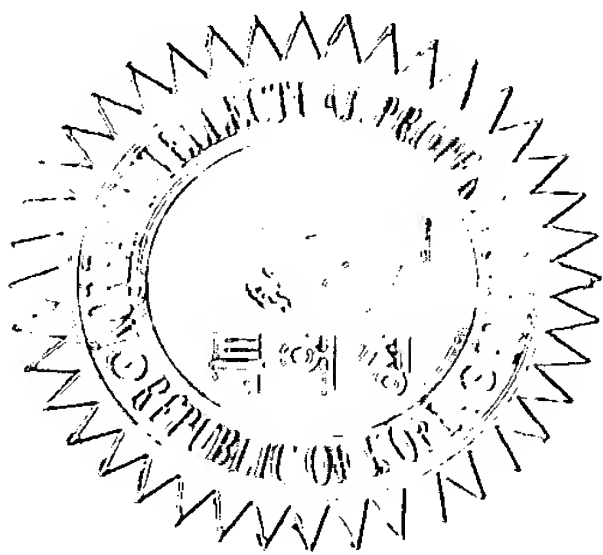
This is to certify that the following application annexed hereto is a true copy from the records of the Korean Intellectual Property Office.

출원 번호 : PCT/KR2001/002310  
Application Number

출원 년 월 일 : 2001년 12월 31일  
Date of Application DEC 31, 2001

출원인 : PROBIONIC CORP.  
Applicant(s)

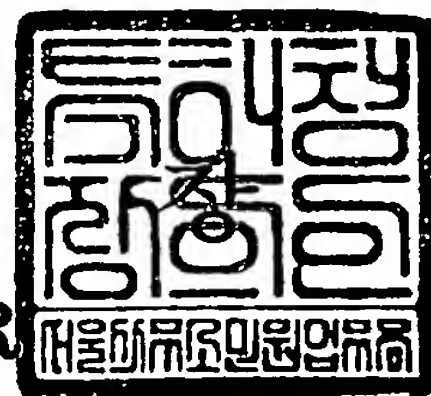
CERTIFIED COPY OF  
PRIORITY DOCUMENT



2005 년 01 월 13 일

특 허 청

COMMISSIONER

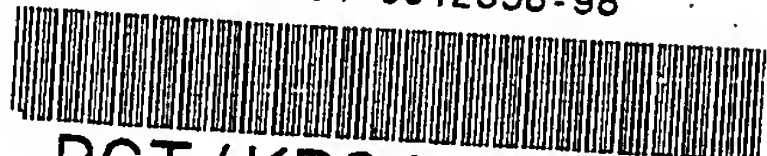


6-1-2001-0012358-98

1/5 ZSA/KR 307

2001-P-03

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PCT/KR01/02310

2001.12.31

수리관청 (이철승)

only

lo.

PCT/KR 01/02310

31 DECEMBER 2001 (31.12.01)

0-3

Name of receiving Office and "PCT International Application"

Korean Intellectual Property Office  
P C T International Application

0-4

Form - PCT/RO/101 PCT Request

0-4-1

Prepared using

PCT-EASY Version 2.92  
(updated 01.03.2001)

0-5

Petition

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty

0-6

Receiving Office (specified by the applicant)

Korean Industrial Property Office  
(RO/KR)

0-7

Applicant's or agent's file reference

2001-P-03

I

Title of invention

NOVEL Lactobacillus reuteri USEFUL AS PROBIOTICS

II

Applicant

II-1

This person is:

applicant only

II-2

Applicant for

all designated States except US

II-4

Name

PROBIONIC CORP.

II-5

Address:

Korea Research Institute of Bioscience & Biotechnology, Eun-dong 52, Yusung-gu  
305-600 Daejeon  
Republic of Korea

II-6

State of nationality

KR

II-7

State of residence

KR

II-8

Telephone No.

82-02-558-4762

II-9

Facsimile No.

82-02-563-4762

II-10

e-mail

bio21@biopatent21.com

\* DELETED BY RO

III-1

Applicant and/or inventor

III-1-1

This person is:

applicant and inventor

III-1-2

Applicant for

US only

III-1-4

Name (LAST, First)

PARK, Yong-HA

III-1-5

Address:

Mokdong APT 1305-106, Shinjung-dong,  
Yangchun-gu  
158-070 Seoul  
Republic of Korea

III-1-6

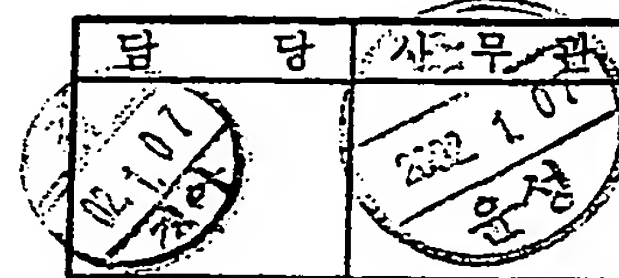
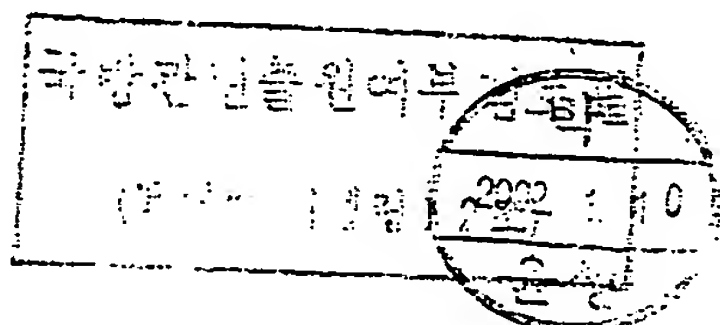
State of nationality

KR

III-1-7

State of residence

KR



## PCT REQUEST

2001-P-03

Original (for SUBMISSION) - printed on 31.12.2001 10:16:59 AM

<b>III-2</b>	<b>Applicant and/or inventor</b>	
III-2-1	This person is:	applicant and inventor
III-2-2	Applicant for	US only
III-2-4	Name (LAST, First)	CHANG, Young-Hyo
III-2-5	Address:	Gubongmaeul APT 803-806, Kwanjeo-dong, Seo-gu 302-723 Daejon Republic of Korea
III-2-6	State of nationality	KR
III-2-7	State of residence	KR
<b>III-3</b>	<b>Applicant and/or inventor</b>	
III-3-1	This person is:	applicant and inventor
III-3-2	Applicant for	US only
III-3-4	Name (LAST, First)	LEE, In-Sun
III-3-5	Address:	Samwhan APT 3-110, Guwoon-dong, Kweonsun-gu 441-341 Suwon Republic of Korea
III-3-6	State of nationality	KR
III-3-7	State of residence	KR
<b>III-4</b>	<b>Applicant and/or inventor</b>	
III-4-1	This person is:	applicant and inventor
III-4-2	Applicant for	US only
III-4-4	Name (LAST, First)	YOON, Jung-Hoon
III-4-5	Address:	Eun-dong 52, Yusung-gu 305-333 Daejon Republic of Korea
III-4-6	State of nationality	KR
III-4-7	State of residence	KR
<b>III-5</b>	<b>Applicant and/or inventor</b>	
III-5-1	This person is:	applicant and inventor
III-5-2	Applicant for	US only
III-5-4	Name (LAST, First)	KIM, Chul-Joong
III-5-5	Address:	Hanwool APT 103-801, Shinsung-dong, Yusung-gu 305-707 Daejon Republic of Korea
III-5-6	State of nationality	KR
III-5-7	State of residence	KR

## PCT REQUEST

2001-P-03

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IV-1	<b>Agent or common representative; or address for correspondence</b> The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name (LAST, First)	LEE, Young-Ryeol
IV-1-2	Address:	LEE & GENE Patent & Law Office, Chungwoon Tower Villa B-105, Yeoksam-dong, Kangnam-gu 135-080 Seoul Republic of Korea
IV-1-3	Telephone No.	82-02-558-4762
IV-1-4	Facsimile No.	82-02-563-4762
IV-1-5	e-mail	bio21@biopatent21.com
V	<b>Designation of States</b>	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AP: GH GM KE LS MW MZ SD SL SZ TZ UG ZW and any other State which is a Contracting State of the Harare Protocol and of the PCT EA: AM AZ BY KG KZ MD RU TJ TM and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT EP: AT BE CH&LI CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE TR and any other State which is a Contracting State of the European Patent Convention and of the PCT OA: BF BJ CF CG CI CM GA GN GQ GW ML MR NE SN TD TG and any other State which is a member State of OAPI and a Contracting State of the PCT
V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH&LI CN CO CR CU CZ DE DK DM DZ EC EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NO NZ PH PL PT RO RU SD SE SG SI SK SL TJ TM TR TT TZ UA UG US UZ VN YU ZA ZW

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## PCT REQUEST

2001-P-03

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<b>V-5</b>	<b>Precautionary Designation Statement</b> In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.	
<b>V-6</b>	<b>Exclusion(s) from precautionary designations</b>	NONE
<b>VI-1</b>	<b>Priority claim of earlier national application</b>	
VI-1-1	Filing date	07 March 2001 (07.03.2001)
VI-1-2	Number	2001-11797
VI-1-3	Country	KR
<b>VI-2</b>	<b>Priority document request</b> The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s):	VI-1 ] <sup>*</sup>
<b>VII-1</b>	<b>International Searching Authority Chosen</b>	Korean Industrial Property Office (KIPO) (ISA/KR)
<b>VIII</b>	<b>Declarations</b>	Number of declarations
VIII-1	Declaration as to the identity of the inventor	-
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	-
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	-
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-

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## PCT REQUEST

2001-P-03

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IX	Check list	number of sheets	electronic file(s) attached
IX-1	Request (including declaration sheets)	5	-
IX-2	Description (excluding sequence listing part)	19	-
IX-3	Claims	1	-
IX-4	Abstract	1	EZABST00.TXT
IX-5	Drawings	0	-
IX-7a	Sub-total number of sheets	26	
IX-6a	Sequence listing part of description filed in computer readable form in addition to being filed in paper form (under Section 801(a)(ii))	2	[Diskette] <sup>Δ</sup>
IX-7	TOTAL	28	
	<b>Accompanying items</b>	<b>paper document(s) attached</b>	<b>electronic file(s) attached</b>
IX-8	Fee calculation sheet	✓	-
IX-16	Sequence listing in computer readable form:		[1 Diskette] <sup>ΔΔΔ</sup>
IX-16 (-ii)	additional copies including, where applicable, the copy for the purposes of international search under Rule 13ter	-	
IX-17	PCT-EASY diskette	-	Diskette
IX-19	Figure of the drawings which should accompany the abstract		
IX-20	Language of filing of the international application	English	
X-1	Signature of applicant, agent or common representative		
X-1-1	Name (LAST, First)	LEE, Young-Ryeol	

Δ DELETED  
BY ROΔΔΔ  
ADDED  
BY RO

## FOR RECEIVING OFFICE USE ONLY

10-1	Date of actual receipt of the purported international application	31 DECEMBER 2001 (31.12.01)
10-2	Drawings:	
10-2-1	Received	
10-2-2	Not received	
10-3	Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application	
10-4	Date of timely receipt of the required corrections under PCT Article 11(2)	
10-5	International Searching Authority	ISA/KR
10-6	Transmittal of search copy delayed until search fee is paid	

## FOR INTERNATIONAL BUREAU USE ONLY

11-1	Date of receipt of the record copy by the International Bureau	
------	--	--

[seperat signed paper of attorney attached] <sup>ΔΔΔ</sup>ΔΔΔ  
ADDED  
BY RO

PCT

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0-1	<b>Form - PCT/RO/134 (EASY)</b> <b>Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)</b>	
0-1-1	Prepared using	PCT-EASY Version 2.92 (updated 01.03.2001)
0-2	<b>International Application No.</b>	<b>PCT/KR 01/02310</b>
0-3	<b>Applicant's or agent's file reference</b>	2001-P-03
1	<b>The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:</b>	
1-1	page	9
1-2	line	27-31
1-3	<b>Identification of Deposit</b>	
1-3-1	Name of depositary institution	Korean Culture Center of Microorganisms
1-3-2	Address of depositary institution	361-221, Yurim B/D, Honje 1, Sudaemun, Seoul, 120-091, Republic of Korea
1-3-3	Date of deposit	02 October 2000 (02.10.2000)
1-3-4	Accession Number	KCCM 10214
1-4	<b>Additional Indications</b>	NONE
1-5	<b>Designated States for Which Indications are Made</b>	all designated States
1-6	<b>Separate Furnishing of Indications</b> These indications will be submitted to the International Bureau later	NONE
2	<b>The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:</b>	
2-1	page	18
2-2	line	12-14
2-3	<b>Identification of Deposit</b>	
2-3-1	Name of depositary institution	Korean Culture Center of Microorganisms
2-3-2	Address of depositary institution	361-221, Yurim B/D, Honje 1, Sudaemun, Seoul, 120-091, Republic of Korea
2-3-3	Date of deposit	02 October 2000 (02.10.2000)
2-3-4	Accession Number	KCCM 10214
2-4	<b>Additional Indications</b>	NONE
2-5	<b>Designated States for Which Indications are Made</b>	all designated States
2-6	<b>Separate Furnishing of Indications</b> These indications will be submitted to the International Bureau later	NONE



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2001-P-03

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0-4	This form was received with the international application: (yes or no)	
0-4-1	Authorized officer	

## FOR INTERNATIONAL BUREAU USE ONLY

0-5	This form was received by the international Bureau on:	
0-5-1	Authorized officer	

**PCT (ANNEX - FEE CALCULATION SHEET)**

2001-P-03

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(This sheet is not part of and does not count as a sheet of the international application)

<b>0</b>	<b>For receiving Office use only</b>			
<b>0-1</b>	International Application No.	<b>PCT/KR 01 / 02310</b>		
<b>0-2</b>	Date stamp of the receiving Office	<b>31.12.2001.</b>		
<b>0-4</b>	<b>Form - PCT/RO/101 (Annex)</b>			
<b>0-4-1</b>	PCT Fee Calculation Sheet Prepared using	<b>PCT-EASY Version 2.92 (updated 01.03.2001)</b>		
<b>0-9</b>	<b>Applicant's or agent's file reference</b>		<b>2001-P-03</b>	
<b>2</b>	<b>Applicant</b>		<b>PROBIONIC CORP., et al.</b>	
<b>12</b>	<b>Calculation of prescribed fees</b>	fee amount/multiplier	total amounts (KRW)	
<b>12-1</b>	Transmittal fee <b>T</b>	⇒	<b>45,000</b>	
<b>12-2</b>	Search fee <b>S</b>	⇒	<b>150,000</b>	
<b>12-3</b>	International fee			
	Sub-total number of sheets	<b>26</b>		
	Basic fee (first 30 sheets) <b>b1</b>	<b>500,000</b>		
<b>12-4</b>	Remaining sheets	<b>0</b>		
<b>12-5</b>	Additional amount <b>(X)</b>	<b>0</b>		
<b>12-6</b>	Total additional amount <b>b2</b>	<b>0</b>		
<b>12-4a</b>	Additional component	<b>400</b>		
<b>12-5b</b>	Additional amount <b>(X)</b>	<b>0</b>		
<b>12-6c</b>	Total additional component <b>b3</b>	<b>0</b>		
<b>12-7</b>	<b>b1 + b2 + b3 = B</b>	<b>500,000</b>		
<b>12-8</b>	Designation fees			
	Number of designations contained in international application	<b>89</b>		
<b>12-9</b>	Number of designation fees payable (maximum 6)	<b>6</b>		
<b>12-10</b>	Amount of designation fee <b>(X)</b>	<b>108,000</b>		
<b>12-11</b>	Total designation fees <b>D</b>	<b>648,000</b>		
<b>12-12</b>	PCT-EASY fee reduction <b>R</b>	<b>-154,000</b>		
<b>12-13</b>	Total International fee (B+D-R) <b>I</b>	⇒	<b>994,000</b>	
<b>12-14</b>	Fee for priority document			
	Number of priority documents requested	<b>1</b>		
<b>12-15</b>	Fee per document <b>(X)</b>	<b>0</b>		
<b>12-16</b>	Total priority document fee <b>P</b>	⇒	<b>0</b>	
<b>12-17</b>	<b>TOTAL FEES PAYABLE (T+S+I+P)</b>	⇒	<b>1,189,000</b>	
<b>12-19</b>	<b>Mode of payment</b>	<b>cash</b>		

**VALIDATION LOG AND REMARKS**

## PCT (ANNEX - FEE CALCULATION SHEET)

2001-P-03

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13-2-1	Validation messages Request	Green? The title of the invention should preferably be entered in capital letters. Please verify.
13-2-2	Validation messages States	Green? More designations could be made. The following States have not been designated: KR. Please verify.
13-2-5	Validation messages Biology	Green? Biology: Several PCT contracting States require these indications to be included in the description. Please verify.
13-2-7	Validation messages Contents	Yellow! The power of attorney or a copy of the general power of attorney will need to be furnished unless all applicants sign the request form.
		Green? The international application contains no drawings. Please verify.
		Yellow Type of carrier and/or number has not been indicated.
13-2-8	Validation messages Fees	Green? Please confirm that fee schedule utilized is the latest available
		Yellow Fee amount(s) should not equal zero.
13-2-11	Validation messages For receiving Office/International Bureau use only	Green? Verify electronic data for consistency against printed form.

## POWER OF ATTORNEY

Agent(s) (Common Representative)

Name: LEE, Young-Ryeol

Address: Chungwoon Tower Villa B-105, Yeoksam-dong, Kangnam-ku,  
Seoul 135-080, Republic of Korea

I/We, the undersigned, do hereby appoint the above-identified agent(s) (common representative) as my/our agent/common representative to act for me/us in proceedings concerning all of my/our International Application set forth below.

International Application No.: **PCT/KR 01/02310**

Title of Invention: Acid tolerant probiotic *Lactobacillus reuteri* that can suppresses the growth of pathogenic microorganisms and rotavirus

This 24<sup>th</sup> day of October, 2001

Applicant(s)

Name: Probionic Corp.

Address: Korea Research Institute of Bioscience & Biotechnology, Eun-dong  
52, Yusung-gu, 305-600 Daejeon, Korea



## POWER OF ATTORNEY

Agent(s) (Common Representative)

Name: LEE, Young-Ryeol

Address: Chungwoon Tower Villa B-105, Yeoksam-dong, Kangnam-ku,  
Seoul 135-080, Republic of Korea

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International Application No.: **PCT/KR 01 / 02310.**

Title of Invention: Acid tolerant probiotic *Lactobacillus reuteri* that can suppresses the growth of pathogenic microorganisms and rotavirus

This 24<sup>th</sup> day of October, 2001

Applicant(s)

Name: Yong-Ha Park

Address: Mokdong APT 1305-106, Shinjung-dong, Yangchun-gu, 158-070  
Seoul, Korea



## POWER OF ATTORNEY

Agent(s) (Common Representative)

Name: LEE, Young-Ryeol

Address: Chungwoon Tower Villa B-105, Yeoksam-dong, Kangnam-ku,  
Seoul 135-080, Republic of Korea

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International Application No.: **PCT/KR 01 / 02310**

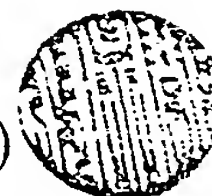
Title of Invention: Acid tolerant probiotic *Lactobacillus reuteri* that can suppresses the growth of pathogenic microorganisms and rotavirus

This 24<sup>th</sup> day of October, 2001

Applicant(s)

Name: Chang, Young-Hyo

(seal)



Address: Gubongmaeul APT 803-806, Kwanjeo-dong, Seo-gu, 302-723  
Daejeon, Korea

## POWER OF ATTORNEY

Agent(s) (Common Representative)

Name: LEE, Young-Ryeol

Address: Chungwoon Tower Villa B-105, Yeoksam-dong, Kangnam-ku,  
Seoul 135-080, Republic of Korea

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International Application No.: **PCT/KR 01/02310**

Title of Invention: Acid tolerant probiotic *Lactobacillus reuteri* that can suppresses the growth of pathogenic microorganisms and rotavirus

This 24<sup>th</sup> day of October, 2001

Applicant(s)

Name: In-Sun Lee

Address: Samwhan APT 3-110, Guwoon-dong, Kweonsun-gu, 441-341  
Suwon, Korea





## POWER OF ATTORNEY

Agent(s) (Common Representative)

Name: LEE, Young-Ryeol

Address: Chungwoon Tower Villa B-105, Yeoksam-dong, Kangnam-ku,  
Seoul 135-080, Republic of Korea

I/We, the undersigned, do hereby appoint the above-identified agent(s) (common representative) as my/our agent/common representative to act for me/us in proceedings concerning all of my/our International Application set forth below.

International Application No.: **PCT/KR 01 / 02310**

Title of Invention: Acid tolerant probiotic *Lactobacillus reuteri* that can suppresses the growth of pathogenic microorganisms and rotavirus

This 24<sup>th</sup> day of October, 2001

Applicant(s)

Name: Jung-Hoon Yoon

Address: Eun-dong 52, Yusung-gu, 305-333 Daejeon, Korea



## POWER OF ATTORNEY

Agent(s) (Common Representative)

Name: LEE, Young-Ryeol

Address: Chungwoon Tower Villa B-105, Yeoksam-dong, Kangnam-kù,  
Seoul 135-080, Republic of Korea

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International Application No.: **PCT/KR 01/02310**

Title of Invention: Acid tolerant probiotic *Lactobacillus reuteri* that can suppresses the growth of pathogenic microorganisms and rotavirus

This 24<sup>th</sup> day of October, 2001

Applicant(s)

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NOVEL *Lactobacillus reuteri* USEFUL AS PROBIOTICS

## TECHNICAL FIELD

5           The present invention relates, in general, to a novel probiotic microorganism and, more particularly, to a *Lactobacillus reuteri* variant from animal sources that inhibits rotavirus infection and other pathogenic microorganisms, as well as being tolerant of gastric and bile acids. Also, this invention relates to a prophylactic and therapeutic composition comprising the same for contributing in  
10 many probiotic ways to the host's general health and preventing and treating diseases or conditions associated with rotavirus and other enteric pathogens.

## BACKGROUND ART

15           Diarrhea is one of the most common infectious diseases in the world. Although various viral, bacterial, and parasitic agents are suspected of causing acute infectious diarrhea and gastroenteritis, rotaviruses have been identified as the most important viral agent of diarrhea and gastroenteritis, e.g., in children and young animals in both developed and developing countries.

20           Rotaviruses cause 35-50% of severe diarrheal episodes in infants and young children in both developed and developing countries, and are the most important etiological agents of severe diarrhea in this age group. Rotaviruses are also the most important viral agents causing diarrhea in many animals, including  
25 swine units. Diarrhea caused by rotavirus is usually watery to pasty in consistency and may vary considerably in color. Affected pigs are usually depressed, off-feed, and dehydrated. Rotavirus destroys villi in the small intestine, hence, the clinical signs seen in diarrheal outbreaks. Additionally, rotaviruses have been linked with the occurrence of gastroenteritis and the secondary infections by enteric bacteria in  
30 affected animals. That is, rotavirus infections are more difficult to treat when accompanied by secondary bacteria, thereby, enhancing the severity of the outbreak. Treatments for rotavirus are based on supportive treatment that addresses dehydration and starvation that occurs with a rotavirus infection. Treatments with antibiotics are only effective against secondary bacterial infections, and

disadvantageous in that antibiotics may remain in the meat. Vaccination may be effective for the prevention of rotaviral infection, but may generate side effects.

5 Bacteria residing in the intestine may also cause diseases in the colonized host such as diarrhea in the intestines. A wide variety of microorganisms exist in the intestines of humans and animals, constituting intestinal microflora. They include bacteria that are not only beneficial to hosts, like lactic acid bacteria, but also some which are potentially or directly harmful to hosts, like *E. coli*, *Salmonella*, *Staphylococcus*, etc. Changes in the external environment, such as increased stress, 10 infection of harmful microorganisms, and the like, may upset the normal balance of the intestinal microflora, resulting in the predominance of harmful microorganisms over beneficial ones. A shift in microflora can lead to abnormal health states of the host, such as diarrhea and, at the worst, even to death. When numbers of harmful microbes reach high levels in the intestine, the host is usually treated by the 15 administration of antibiotics. Although they may be effective for the control of harmful microorganisms, antibiotics are not completely discharged from the host, but partially remain in the host's tissues. Additionally, extended administration of antibiotics induces antibiotic resistance in harmful microorganisms, making it finally impossible to effectively treat diseases they cause. Furthermore, recent legislation 20 in response to environmental concerns stemming from food hygiene has been enacted to substantially lower the acceptable levels of antibiotics present in meats, milks and eggs of livestock. Hence, problems may occur upon use of antibiotics, let alone misuse or abuse thereof.

25 Thus, there is an urgent need to develop methods of treating and preventing infectious diarrhea caused by enteric viruses and microorganisms, and diarrhea associated with antibiotic therapy.

30 There has been an increased interest for microbial species which can beneficially affect the microbial balance of the host, e.g. by producing antimicrobial components or by competitive growing.

35 Probiotics are a class of microorganisms that are defined as live microbial organisms that beneficially affect the animal and human hosts. The beneficial effects include improvement of the microbial balance of the intestinal microflora or by

improving the properties of the indigenous microflora. A better understanding of probiotics in man and animals can be found in the following publication(Fuller R: Probiotics in Man and Animals, J Appl. Bacteriol 1989;66:365-378). Referring to the use of microorganisms in a positive way to benefit health, probiotics are prepared by formulating beneficial microorganisms that inhabit the intestine. Examples of microorganisms available for the preparation of probiotics include aerobes, anaerobes, lactic acid bacteria, and yeasts with lactic acid bacteria being the most popular. Probiotics enjoy the advantage of causing no side effects such as those resulting from the abuse of antibiotics, and inhibiting the abnormal proliferation of harmful microorganisms to maintain normal intestinal flora and to prevent the occurrence of illness. The known benefits of enteral administration of probiotic microorganisms include enhanced host defense to disease, improving colonization resistance of the harmful microflora and numerous other areas of health promotion. Probiotics have been suggested to play an important role in the formation or establishment of a well-balanced, indigenous, intestinal microflora in newborn children or adults receiving high doses of antibiotics.

For the prevention and treatment of diarrhea in livestock or poultry, live bacterial compositions have been developed for use as agents other than antibiotics. In live bacterial compositions, useful live bacteria are directly administered to livestock or poultry, where the bacteria are retained in the intestine of livestock or antagonize enterotoxic bacteria, e.g., *Escherichia coli*, to eliminate the enterotoxic bacteria, during passage of the live bacteria through the intestine. As a result, the enterobacteria microflora is improved so that diarrhea of livestock is prevented and treated. In addition, research results disclose that persistent administration of probiotics to livestock give rise to an increase in feed efficiency and weight gain.

For effective use, probiotics must be resistant to acid, particularly gastric and bile acid, in addition to having inhibitory activity against harmful microorganisms. Because probiotics are usually consumed in specially designed foods that are variously called nutraceuticals or functional foods, they experience acidic environments, first in gastric juice, a strong acid, and then in bile, until arrival at the intestine. In general, bacteria are killed or deactivated by gastric juice and bile acid. Therefore, probiotics must survive gastric juice and bile to reach the intestine, thereby exerting their beneficial functions.

Various kinds of lactic acid bacteria have been already disclosed as probiotics. Lactobacilli have been among the most studied species, and have in certain instances been shown to counteract the proliferation of pathogens. In fact, *Lactobacillus* therapy have increased in recent years with findings that probiotic *Lactobacillus* (a) improves the intersterial microflora, (b) prevents diarrhea, (c) affords protection from colon cancer for human populations, (d) reduces the incidence of experimentally induced large tumors in rats, (e) reduces the fecal concentration of bacterial enzymes known to catalyze the conversion of procarcinogens to proximal carcinogens in humans, and (f) reduces the serum cholesterol levels in swine. In recent, a lactic acid bacterium, identified as *Lactobacillus reuteri* BSA-131, was reported to be tolerant of gastric and bile acids as well as inhibiting the proliferation of harmful microorganisms in the intestine (Chang, et al., Korean J. Appl. Microbiol. Biotechnol., 27, 23-27; Korean Pat. No. 10-211529-0000).

### DISCLOSURE OF THE INVENTION

Leading to the present invention, the intensive and thorough research into a novel strain with more potent probiotic activity, conducted by the present inventors, resulted in the finding that a bacterial strain anaerobically separated from swine excrements has superb inhibitory activity against harmful microorganisms, including rotavirus, with superior tolerance to gastric and bile acids over the conventional *Lactobacillus reuteri* BAS-131 claimed in Korean Pat. No. 10-211529-0000. The novel bacterial strain was identified as a member of *Lactobacillus reuteri*.

So, the invention demonstrates the effectiveness of a novel probiotic lactic acid bacterium in inhibiting the growth of rotavirus and other enteric pathogenic microorganisms, and thereby preventing their infections. The present invention describes lactic acid bacteria isolated from the gastrointestinal tract in pigs and selected by means of, among others, the inhibitory activity against pathogenic microorganisms in vitro and tolerance against acid and bile.



Therefore, it is an object of the present invention to provide a novel enteric bacterial strain, which is tolerant of gastric and bile acids and harmless to humans and animals.

5 It is another object of the present invention to provide probiotics for use in foods, medicines for humans and livestock, and feedstock, which exhibits excellent inhibitory activity against the growth of harmful microorganisms, especially rotavirus, in the intestine.

10 It is a further object of the present invention to provide a method for inhibiting the growth of rotavirus and other harmful microorganisms.

Based on the present invention, the above objects could be accomplished by a provision of *Lactobacillus reuteri* Probio-16 (Accession No. KCCM 10214), inhibitory of the growth of rotavirus and other pathogenic organisms.

15 Other objects, advantages and features of the present invention will become apparent to those skilled in the art from the following discussions.

### BEST MODES FOR CARRYING OUT THE INVENTION

20 It has now been discovered that a novel strain, *Lactobacillus reuteri* Probio-16, which is intestinal bacterium derived from pigs and can grow under anaerobic conditions, has the effect of inhibiting the growth of rotavirus and other microorganisms, thereby preventing and treating diarrhea of mammals, for example livestock, poultry, pet animals, etc.

25 So, the present invention pertains to the identification and characterization of a novel enteric bacterium. Identified as a kind of *Lactobacillus reuteri* species, the novel bacterium of the present invention is characterized to be tolerant of gastric and bile acids.

30 This invention arose from a desire by the inventors to improve on prophylactic and therapeutic methods for treating diarrheal conditions associated with rotavirus infection in mammals, especially in humans and swines. More particularly, the present invention provides an effective and potent agent for the  
35 treatment of diarrhea and/or gastroenteritis, associated with a variety of conditions



linked to rotavirus and enteric bacteria infections. The microorganism of this invention prevents and retards rotavirus infections and is effective in the treatment of diarrhea. Thus, the probiotic strain of the present invention be used for reducing the number of enteric pathogenic bacteria in food items, and in the gastrointestinal tracts of animals.

In fact, it was found that the microorganism of the present invention, beside adhesion to intestinal cells often superior to the reference strains, also had the following characteristic features:

- ability to inhibit the growth of rotavirus and intestinal pathogens;

- ability to grow under a variety of conditions, both in aerobiosis and anaerobiosis, and at different pH values; these properties confer good capacity to adapt to the variable physiological and pathological situations that are met during the transit in the gastrointestinal tract;

- production of a lactic acid;

- high resistance to the gastric and bile acids;

- resistance to lyophilization, without losing viability.

Moreover, the agent of this invention is devoid of the drawbacks and side effects of other known therapies.

A "probiotic" is understood to be a live or dead microbial food supplement which beneficially affects the animal, including human host by improving the individual's microbial balance in the gastrointestinal tract, e.g., *Lactobacillus reuteri* and other *Lactobacillus acidophilus*. That is, a probiotic microorganism is useful for changing the digestive system bacteria in animals when fed orally. For example, a probiotic microorganism, upon oral feeding, can rapidly reduces and replaces the natural or ingested population of bacteria in the digestive system in animals, thereby preventing recurring disorders particularly disorders in the intestine. The probiotic microorganism remains effective in the digestive system for substantial periods of

time even after oral feeding is discontinued.

To search for the probiotic microorganism of the present invention, the following procedures were accomplished:

5 First, 1 g of swine excrements was collected into an anaerobic bag such as that manufactured by BBL, identified as "GasPak pouch", and diluted 10 fold in an anaerobic diluent of pH 2, followed by shaking for 120 min in a shaker. 1 ml of the shaken dilution was spread over a BL agar medium containing 2 % of Oxgal (Difco, trade name) and incubated at 37 °C for 48 hours. After appearance of  
10 colonies on the medium, rod-shaped microorganisms were selected from 144 colonies with monitoring under a microscope and cultured in GAM-semi agar media. The cultures were stored at -80 °C until use.

Then, the selected rods(bacteria) were tested for growth inhibition of harmful microorganisms. In this regard, the following 13 pathogenic species were  
15 used for antibiotic assay according to the Kuroiwa's method (Kuroiwa et al., 1990): *Esherichia coli* KCTC 2441, *Esherichia coli* KCTC 2571, *Klebsiela pneumoniae* KCTC 2208, *Staphylococcus aureus* KCTC 1621, *Staphylococcus epidermis* KCTC 1917, *Salmonella enteritidis* kim sp14, *Shiegella flexneri* KCTC 2008, *Proteus vulgaris* KCTC 2579, *Enterobacter cloacae* KCTC 2361, *Enterococcus lactis*  
20 KCTC 1913, *Serratia marscens* KCTC 2172, *Citrobacter freundii* KCTC 2006, *Bacillus subtilis* KCTC 1021.

After the harmful microorganisms were anaerobically cultured for 18 hours in MRS broths, 1 ml of each culture was spread over MRS agar media. 30 µl of each of the selected bacteria, after being anaerobically cultured for 18 hours in the  
25 same broth, was inoculated onto a paper disc with a diameter of 8 mm. The paper discs were positioned on the MRS plates which were spread with the harmful microorganisms. Inhibitory circles emerging after a certain time period of culturing were measured. The strain which was measured to show the largest inhibitory effect compared with control groups was designated #Probio-16.

30 The selected strains were tested for the growth inhibition of rotavirus as follows. TF-104 monolayer cells were washed twice with phosphate buffered saline and inoculated with rotavirus. After the virus was allowed to adsorb onto the cells for 30 min, the medium was added with serum-free Eagle's Minimum Essential Medium (EMEM) and then with an appropriate amount of trypsin. While  
35 being cultured at 37 °C, the infected cells were observed for cytopathic effect (CPE).

When CPE was observed in about 70 % of the monolayer cells, the MEM medium containing the cells was frozen at  $-70^{\circ}\text{C}$  and thawed. This freeze-thaw procedure was repeated twice. After the centrifugation of the medium, the supernatant was stored at  $-70^{\circ}\text{C}$  until use. Separately, TF-104 monolayer cells were placed on 96-well microplates and washed twice with phosphate buffered saline. After thawing the rotavirus suspension from  $-70^{\circ}\text{C}$ , 10-fold serial dilutions were made. Each dilution was inoculated into 10 wells of the TF-104 monolayer cells, which were then cultured for 72 hours at  $37^{\circ}\text{C}$  in a humidified atmosphere containing 5%  $\text{CO}_2$  while observing CPE. Based on the observation results, the virus existing in the culture was quantitatively determined according to the Reed & Muench's method (Microbiology 3<sup>rd</sup> Edition, Davis et al., Harper & Row Publishers, 1980).

In that way, the inventors have shown that #Probio-16 inhibits the in vitro infection of cells by rotaviruses as well as diarrhea and/or gastroenteritis induced by rotaviruses in an animal model. In one example of the present invention, the antiviral activity assay started with the treatment of a rotaviral culture with trypsin at  $37^{\circ}\text{C}$  for 1 hour. The pre-treated culture was diluted with a serum-free Eagle's Minimum Essential Medium to virus titers of 1.0  $\text{TCID}_{50}/0.1\text{ ml}$ , 10.0  $\text{TCID}_{50}/0.1\text{ ml}$ , 100  $\text{TCID}_{50}/0.1\text{ ml}$ , and 1,000  $\text{TCID}_{50}/0.1\text{ ml}$ . Separately, TF-104 monolayer cells were aliquoted into wells of 96-well microplates and washed twice with phosphate buffered saline. 4 wells were allocated to one culture sample of #Probio-16. Into 4 wells, 1.0  $\text{TCID}_{50}/0.1\text{ ml}$ , 10  $\text{TCID}_{50}/0.1\text{ ml}$ , 100  $\text{TCID}_{50}/0.1\text{ ml}$ , and 1000  $\text{TCID}_{50}/0.1\text{ ml}$  were added in an amount of 90  $\mu\text{l}$ , respectively, immediately followed by the addition of 10  $\mu\text{l}$  (10 %) of the lactobacillus culture. The cells were observed for cytopathic effect at 24, 48 and 72 hours while being cultured at  $37^{\circ}\text{C}$  in a humidified atmosphere containing 5%  $\text{CO}_2$ . The identification of CPE was regarded as absence of antiviral activity.

Antibiotic susceptibility of #Probio-16 was assayed according to Microbiology procedures handbook vol. 1 (Henry D. Isenberg, ASM) and Korean Pat. Publication No. 91-4366. In this regard, 13 antibiotics, that is, cephalaxcin, erythromycin, flumequinine, furazolidine, gentamycin, procaine, penicillin G, norflaxacin, spectinomycin, tetracycline, tiamuline, neomycin, chloramphenicol, and kanamycin, were dissolved in appropriate solvents. By concentrations, the prepared antibiotic solutions were inoculated in an amount of 30  $\mu\text{l}$  onto a paper disc

which was then allowed to stand for 1 hour at 4 °C. Inhibitory circles emerging after 24 hours of anaerobic incubation were measured.

5 The identification of *Lactobacillus reuteri* strain is confirmed using standard microbiological and physicochemical tests, the taxonomic characteristics of the species and 16S rRNA sequencing.

10 With regard to the morphological and physicochemical characteristics of #Probio-16, it is a Gram-positive bacterium which can grow in both aerobic and anaerobic conditions without formation of spores, and has no motility. Its optimal temperature ranges from 30 to 37 °C. This bacterium does not generate gas and indole, nor show hemolysis, nor reduce nitric acid. It cannot decompose urea and proteins, but ferments mannose and raffinose and is tolerant of 5 % bile acid. This  
15 bacterium is positive for arginine dehydrolase, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, leucine arylamidase, phenylalanine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, serine arylamidase, alpha-galactosidase, alpha-glucosidase, beta-glucosidase, alpha-arabinosidase, and beta-glucuronidase; negative for catalase, lipase, lecithinase, alkali phosphatase, glutamate decarboxylase, pyro glutamate  
20 arylamidase, glutamyl glutamate arylamidase, 6-phosphate-beta-galactosidase, beta-N-acetyl-glucosidase, and alpha-fructosidase.

The 16S rDNA of #Probio-16 has the nucleotide sequence listed in SEQ. ID. NO. 1. Through a molecular systematic analysis based on 16S rDNA  
25 sequences, #Probio-16 was found to have 99.5% homology with the type strain of *Lactobacillus reuteri*, showing the highest phylogenetic relatedness among the strains tested. Based on these results, #Probio-16 was identified as a member of *Lactobacillus reuteri* strain and deposited at the Korean Culture Center of Microorganisms under accession No. KCCM 10214 on Oct. 2, 2000 in accordance  
30 with the terms and provisions of the Budapest Treaty relating to deposit of microorganisms.

Also, the present invention is directed to a method for inhibiting harmful microorganisms using the novel strain. In the method, the inhibitory activity of

*Lactobacillus reuteri* Probio-16 against the growth of harmful microorganisms is optimally effected at 30 to 70 °C.

An experiment for antibiotic susceptibility showed that *Lactobacillus reuteri* Probio-16 is susceptible to cephalexcin with a minimal inhibitory concentration (MIC) of 90 µg/ml, erythromycin with an MIC of 4 µg/ml, flumequinine with an MIC of 4,000 µg/ml, furazolidine with an MIC of 90 µg/ml, gentamycine with an MIC of 100 µg/ml, penicillin G with an MIC of 4, norflaxacine with an aid of 100, spectinomycine with an MIC of 2,500 µg/ml, tetracycline with an MIC of 1,500 µg/ml, tiamuline with an MIC of 300 µg/ml, neomycine with an MIC of 100 µg/ml, chloramphenicol with an MIC of 200 µg/ml, and kanamycine with an MIC of 4,000 µg/ml,

For stable long preservation, the microorganism of the present invention is stored at -80 °C in glycerol or freeze-dried from a suspension in 10 % non-fat milk.

In addition, the present invention is directed to foods, medicines for humans and animals, and feedstock, which contain the novel microorganism. The microorganism of the present invention may be used alone, with a carrier or as an additive to a foodstuff, or in other compositions suitable for human and livestock consumption. That is, the microorganism of the present invention is useful because it can be added both to foodstuffs that do not contain probiotic bacteria (for the purpose of giving these products a probiotic value as well) and to foodstuffs already containing some probiotic bacteria (for the purpose of enhancing and/or completing their probiotic value).

A pharmaceutical, veterinary or alimentary composition of the present invention comprises the isolated *Lactobacillus reuteri* Probio-16 alone as a probiotic lactic acid bacteria or a mixture of two or more of strains mixed with an appropriate vehicle (carrier). For example, Other preferred probiotic strains that may be used in accordance with the composition of the present invention may be selected from one or more microorganisms suitable for human or animal consumption and which is able to improve the microbial balance in the human or animal intestine. Examples of suitable probiotic microorganisms include yeasts such as *Saccharomyces*, *Candida*, *Pichia* and *Torulopsis*, moulds such as *Aspergillus*, *Rhizopus*, *Mucor*, and *Penicillium* and bacteria such as the genera *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Leuconostoc*, *Bacteroides*, *Staphylococcus*, *Lactococcus*, *Bacillus*,



*Streptococcus*, *Fusobacterium*, *Propionibacterium*, *Enterococcus*, *Pediococcus*, and *Micrococcus*. Specific examples of suitable probiotic micro-organisms are: *Saccharomyces cerevisiae*, *Bacillus coagulans*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium longum*,  
5 *Enterococcus faecium*, *Enterococcus faecalis*, *Lactobacillus acidophilus*, *Lactobacillus alimentarius*, *Lactobacillus casei*, *Lactobacillus curvatus*, *Lactobacillus delbruckii*, *Lactobacillus johnsonii*, *Lactobacillus farciminus*, *Lactobacillus gasseri*, *Lactobacillus helveticus*, *Lactobacillus rhamnosus*, *Lactobacillus sake*, *Lactococcus lactis*, *Micrococcus varians*, *Pediococcus acidilactici*, and *Staphylococcus xylosus*. Other agents, for instance, that may be  
10 added to the composition for this particular application are bulking agents, carbon black, high fiber additives, encapsulation agents, protease inhibitors, glycosidase inhibitors, and carrier lipids, optionally micellar, among others. These may be present in amounts known in the art. In the composition the isolated *Lactobacillus reuteri* Probio-16 is present in lyophilized form or in the form of capsules, solutions  
15 or drinkable suspensions or powder. Also, the probiotic microorganisms are preferably in powdered, dried form; especially in spore form. Further, if desired, the probiotic microorganism may be encapsulated to further increase the probability of survival.

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These compositions can be administered orally or mixed with feedstock or food products such as milk, yoghurt or milk-products, juices, cereals, chewing gum, crackers, candies, vitamin supplements, meats, vegetables and fruits, blended or otherwise as baby food for example, and cookies, for the treatment or prophylaxis of  
25 gastrointestinal pathologies in which it is desirable to administer lactobacilli, as for example in the case of intestinal diarrhea of various origins, ulcerative colitis and related pathologies.

In another embodiment of the present invention, instead of composition the prepared cells, homogenates thereof or fractions containing the cell wall  
30 components, also may be given orally to livestock, poultry, pet animals, etc. in the form of a liquid, generally an aqueous liquid; or if necessary and desired, the microorganism may be dried to a powdery form, which is added to feed for livestock, poultry, pets, etc.

Usable instead of conventional antibiotics, the foods, medicines and feedstock of the present invention comprising the novel strain inhibit harmful intestinal microorganisms to maintain a stable balance in human and animal intestinal flora, thereby benefiting the health of humans and bringing about an improvement in weight gain, meat quality, milk production, and immunity in livestock. That is, the compositions of the present invention can also be administered in consequence of antibiotic treatments in order to preserve the non-pathological intestinal bacterial flora. Also, it will be understood that the composition of feedstuff of this invention achieves excellent results in increasing rate of weight, increased weight per a day and feed efficiency and is preferred by various domesticated animals, such as cows, swines, dogs, chickens, and etc.

Moreover, the novel anti-rotaviral microorganism of this invention is suitable for use in most instances of rotavirus infection, and particularly in cases where other therapies are either ineffective or clinically contraindicated.

So, this invention also provides a method of retarding the onset of, or countering, rotavirus infection of a subject's cells comprising administering to a subject at risk for, or suffering from, rotavirus infection an anti-rotavirus effective amount of the microorganism of this invention or mixtures thereof, or a composition comprising the microorganism of the invention and/or a pharmaceutically-acceptable carrier and/or a foodstuff and/or other additives as described above. The composition may incorporate other anti-viral or anti-microbial agents, as suitable for effective treatment of a rotavirus infection taking into account the age, general health, and nutritional status of the subject. Thus, the present invention finds its application in the prevention and treatment of diseases such as diarrhea and gastroenteritis caused by rotavirus and associated microorganisms.

The microorganism of this invention exhibits additional advantages for the treatment of livestock since, as already indicated, it is normal intestinal microflora. The present microorganism is thus unlikely to elicit toxic, immunological or allergic reactions in treated subjects.

In the present invention, livestock includes swine, cow, horse, goat, sheep, etc.; poultry includes fowls such as chicken, etc.; and pets include dogs, cats, etc.



A better understanding of the present invention may be obtained in light of the following examples which are set forth to illustrate, but are not to be construed to limit the present invention.

5      EXAMPLE 1: Isolation of Bacteria Tolerant of Gastric and Bile Acids

From healthy, 11 month-old female swine, excrements were anaerobically collected into a GasPak pouch (BBL). 1 g of the swine excrements was diluted 1:10 with an anaerobic diluent (a solution 0.78%  $K_2HPO_4$ , salt mix 37.5 ml, L-cystein 0.5 g, 25 % L-ascorbic acid 2 ml, 8 %  $Na_2CO_3$  50 ml, and 0.1 % resazurin 1 ml in distilled sterile water 860 ml, added with agar 0.5 g) adjusted to pH 2, followed by shaking for 120 min in a shaker. 1 ml of the shaken dilution was spread over a BL agar medium containing 2 % of Oxgal (Difco) and incubated at 37 °C for 48 hours. After observation of the colonies appeared on the medium with a microscope, 144 rod-shaped bacteria were selected, and cultured in GAM-semi agar media. The cultures were stored at -80 °C until use.

EXAMPLE 2 : Determination of Anti-rotaviral Activity

After being washed twice with phosphate buffered saline, TF-104 monolayer cells were inoculated with rotavirus. The rotavirus was allowed to adsorb onto the cells for 30 min, and the medium was added with serum-free Eagle's Minimum Essential Medium (EMEM) and then with an appropriate amount of trypsin. During incubation at 37 °C, the infected cells were observed for cytopathic effect (CPE). When CPE was observed in about 70 % of the monolayer cells, the process of freezing at -70 °C and thawing of the cell culture was repeated twice. After the centrifugation of the cell culture, the supernatant, which was free of the cells, was stored at -70 °C until use. Separately, TF-104 monolayer cells were placed on 96-well microplates and washed twice with phosphate buffered saline. The rotavirus suspension stored at -70 °C was thawed and 10-fold serial dilutions were made. Each dilution was inoculated into 10 wells of the TF-104 monolayer cells, which were then cultured for 72 hours at 37 °C in a humidified atmosphere containing 5%  $CO_2$  while observing CPE. Based on the observation results, the virus existing the culture was quantitatively determined according to the

Reed & Muench's method (Microbiology 3<sup>rd</sup> Edition (Davis et al., Harper & Row Publishers, 1980)).

For the antiviral activity assay, a rotaviral culture was trypsinized at 37 °C for 1 hour and diluted with a serum-free Eagle's minimum essential medium to virus titers of 1.0 TCID<sub>50</sub>/0.1 ml, 10.0 TCID<sub>50</sub>/0.1 ml, 100 TCID<sub>50</sub>/0.1 ml, and 1,000 TCID<sub>50</sub>/0.1 ml. Separately, TF-104 monolayer cells were aliquoted into wells of 96-well microplates and washed twice with phosphate buffered saline. 4 wells were allocated to one culture sample of the isolated strain. Into 4 wells, 1.0 TCID<sub>50</sub>/0.1 ml, 10 TCID<sub>50</sub>/0.1 ml, 100 TCID<sub>50</sub>/0.1 ml, and 1000 TCID<sub>50</sub>/0.1 ml were added in an amount of 90 µl, respectively, immediately followed by the addition of 10 µl (10 %) of the bacterial culture. While being cultured at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>, the cells were observed for cytopathic effect at 24, 48 and 72 hours. If any CPE was observed, the culture was regarded as having no antiviral activity.

The inhibitory effects of the selected strain on rotavirus is given in Table 1, below.

TABLE 1

Inhibitory activity of the Selected Strain(#Probio-16) Against Rotavirus

	1000 TCID <sub>50</sub>	100 TCID <sub>50</sub>	10 TCID <sub>50</sub>	1 TCID <sub>50</sub>
24 hrs after incubation	-	-	-	-
48 hrs after incubation	+	-	-	-
72 hrs after incubation	+	±	-	-

### EXAMPLE 3: Test for Inhibitory Activity of the Isolated Strain Against Growth of Harmful Microorganisms

The selected strain were tested for growth inhibition of harmful microorganisms according to the Kuroiwa's method (Kuroiwa et al., 1990), using the following 13 microorganism species that are usually used for antibiotic assay: . After the harmful microorganisms were anaerobically cultured for 18 hours in MRS broths (Difco), 1 ml of each culture was spread over MRS agar media. Afterwards,

30  $\mu$ l of each of the selected bacilli, after being anaerobically cultured for 18 hours in the same broth, was inoculated onto a paper disc with a diameter of 8 mm. The paper discs were positioned on the harmful microorganism-coated MRS plates. Inhibitory circles emerging after a certain time period of culturing were measured.

5 The strain which was measured to show the largest inhibitory effect compared with control groups was called #Probio-16.

The inhibitory activity of #Probio-16 against harmful microorganisms is given in Table 2, below.

TABLE 2

## Inhibitory Activity of #Probio-16 Against Microorganisms

Harmful Microorganisms	Inhibitory Activity(diameter, mm)
<i>Esherichia coli</i> KCTC 2441	15
<i>Esherichia coli</i> KCTC 2571	17
<i>Klebsiela pneumoniae</i> KCTC 2208	15
<i>Staphylococcus aureus</i> KCTC 1621	16
<i>Staphylococcus epidermis</i> KCTC 1917	18
<i>Salmonella enteritidis</i> kim sp14	16
<i>Shiegella flexneri</i> KCTC 2008	16
<i>Proteus vulgaris</i> KCTC 2579	15
<i>Enterobacter cloacae</i> KCTC 2361	18
<i>Enterococcus lactis</i> KCTC 1913	15
<i>Serratia marscens</i> KCTC 2172	15
<i>Citrobacter freundii</i> KCTC 2006	15
<i>Bacillus subtilis</i> KCTC 1021	16

As apparent from the data, #Probio-16 is identified as being very effective, with similar inhibitory activity versus the 13 microorganism species. Additionally, #Probio-16 was found to be more inhibitory of harmful microorganisms than the preexisting *Lactobacillus reuteri* strain (BSA-131) disclosed in Chang et al., Kor. J. Appl. Microbiol. Biotechnol., 27, 23-27; Korean Pat. No. 10-211529-0000. Also,

15 the novel strain of the present invention was measured to be more viable than the conventional one.

## EXAMPLE 4: Susceptibility of #Probio-16 to Antibiotics

Assay of #Probio-16 for antibiotic susceptibility was conducted according to Microbiology Procedures Handbook vol. 1 (Henry D. Isenberg, ASM) and Korean

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Pat. Publication No. 91-4366, with regard to 13 antibiotics, including cephalalexin, erythromycin, flumequinine, furazolidine, gentamycin, procaine, penicillin G, norflaxacin, spectinomycin, tetracycline, tiamuline, neomycin, chloramphenicol, and kanamycin. By concentrations, antibiotic solutions were inoculated in an amount of 30  $\mu$ l onto a paper disc which was then allowed to stand for 1 hour at 4 °C. Inhibitory circles emerging after 24 hours of anaerobic incubation were measured. The results are given in Table 3, below.

TABLE 3

## Antibiotic Susceptibility of #Probio-16

Antibiotics	MIC(ug/ml)
Cephalexin	90
Erythromycin	4
Flumequinine	4000
Furazolidine	90
Gentamycin	100
procaine Penicilline G	4
Norflaxacin	1000
Spectinomycin	2500
Tetracycline	1500
Tiamuline	300
Neomycin	100
Chloramphenicol	200
Kanamycin	4000

## 10 EXAMPLE 5: Identification of #Probio-16

Strain #Probio-16 isolated in Example 1 was cultured at 37 °C in MRS media (Difco). To identify #Probio-16, its morphology and physicochemistry were characterized according to the Yoon's method (Yoon et al., Int. J. Syst. Bacteriol., 47, 904, 1997) with the aid of API 32A and CHL kits (Biomerio) and its 16S rDNA were sequenced and analyzed according to the Yoon's method (Yoon et al., Int. J. Syst. Bacteriol., 47, 933, 1997).

Morphological and physicochemical characteristics of #Probio-16 are summarized in Table 4, below.

TABLE 4

## Morphological and Physicochemical Characteristics of #Probio-16

Characteristics	Results
Aerotolerance	+
Catalase	-
Hemolysis	-
OF test	F
Motility	-
Gas formation	-
Spore formation	-
Lipase	-
Lecithinase	-
Protein degradation	-
Alkaline phosphatase	-
Urease	-
Indole formation	-
Nitrate reduction	-
Alginine dehydrolase	+
Alginine aryamidase	+
Proline aryamidase	+
Leucyl glycine aryamidase	+
Leucine arylamidase	+
Phenylalanine arylamidase	+
Tyrosine arylamidase	+
Alanine arylamidase	+
Glycine arylamidase	+
Histidine arylamidase	+
Serine arylamidase	+
Glutamate decarboxylase	-
Pyro glutamate arylamidase	-
Glutamyl glutamate arylamidase	-
Alpha-galactosidase	+
Beta-galactosidase	+
6-Phosphate-beta-galactosidase	-
Alpha-glucosidase	+
Beta-glucosidase	+
Alpha-arabinosidase	+
Beta-glucuronidase	+
Beta-N-acetyl-glucosaminidase	-
Alpha-Fructosidase	-
Mannose fermentation	+
Raffinose fermentation	+
Resistance to bile acids(5%)	+
+ positive; - negative	

As seen in Table 4, #Probio-16 is a Gram-positive bacterium which can grow in both aerobic and anaerobic conditions without formation of spores, and has

no motility. Its optimal growth temperature ranges from 30 to 37 °C. This strain does not generate gas and indole, nor show hemolysis, nor reduce nitric acid. It cannot decompose urea and proteins, but ferments mannose and raffinose and is tolerant of 5 % bile acid and positive for alginine dehydrolase, alginine arylamidase, and proline arylamidase.

The 16S rDNA of #Probio-16 has the nucleotide sequence listed in SEQ. ID. NO. 1.

Through a molecular systematic analysis based on 16S rDNA sequences, #Probio-16 was found to have 99.5% homology with the type strain of *Lactobacillus reuteri*, showing the highest phylogenetic relatedness among the strains tested. Also, it was found that #Probio-16 is different from the preexisting probiotic BSA-131 in two nucleotide sequences. Based on these results, #Probio-16 was identified as a novel *Lactobacillus reuteri* strain and deposited in the Korean Culture Center of Microorganisms under accession No. KCCM 10214 on Oct. 2, 2000.

#### INDUSTIRAL APPLICABILITY

As described hereinbefore, the novel strain of the present invention has inhibitory activity against rotavirus and pathogenic bacteria, as well as being tolerant of gastric and bile acid. Useful as a probiotic which can substitute for conventional antibiotics, therefore, the novel strain of the present invention inhibits harmful intestinal microorganisms to maintain a stable balance in human and animal intestinal flora, thereby benefiting the health of humans and bringing about an improvement in weight gain, meat quality, milk production, and immunity in livestock.

The present invention has been described in an illustrative manner, and it is to be understood that the terminology used is intended to be in the nature of description rather than of limitation. Many modifications and variations of the present invention are possible in light of the above teachings. Therefore, it is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

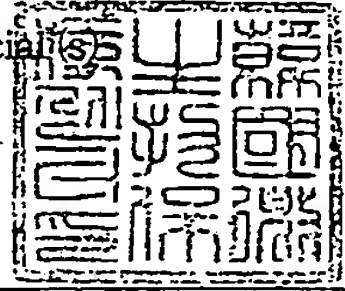


19  
BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To. Yong-Ha Park  
Mokdong Apt. 1302-1507, #327,  
Shinjung-dong, Yangchun-ku,  
Seoul, 158-070  
Korea

RECEIPT IN THE CASE OF AN ORIGINAL  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR :  <i>Lactobacillus reuteri</i> Probio-16	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  KCCM-10214
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on Sep. 20. 2000. (date of the original deposit) <sup>1</sup>	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms  Address : 361-221, Yurim B/D Hongje-1-dong, Seodaeunmun-gu SEOUL 120-091 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of of authouized official(s)  Date: Oct. 2. 2000. 

<sup>1</sup> Where Rule 5.4(d) applies, such date is the date on which the status of international depositary authority was acquired : where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.



## CLAIM

1. *Lactobacillus reuteri* Probio-16 (Accession No. KCCM 10214), inhibitory of the growth of rotavirus and other pathogenic organisms.  
5
2. A pharmaceutical, veterinary, or alimentary composition comprising an effective amount of probiotic *Lactobacillus reuteri* Probio-16 as defined in claim 1 and a carrier.
- 10 3. The composition of claim 2 wherein the composition is useful for changing the digestive system bacteria in mammals including humans, when fed orally.
4. The composition of claim 2 wherein the composition is useful for the prevention and treatment of diarrhea in mammals including humans, when fed orally.  
15
5. A feedstock or food composition including an effective amount of the probiotic composition as defined in claim 2.
- 20 6. A method for reducing the incidence of or treating infectious diarrhea in mammals, including human, said method comprising enterally administering an effective amount of *Lactobacillus reuteri* Probio-16 as defined in claim 1 or the probiotic composition defined in claim 2 to said mammals.
- 25 7. A method for retarding the onset of or treating an infection caused by rotaviruses in mammals, including human, said method comprising enterally administering an effective amount of *Lactobacillus reuteri* Probio-16 as defined in claim 1 or the probiotic composition defined in claim 2 to said mammals.

**ABSTRACT**

5 The present invention relates to a *Lactobacillus reuteri* variant from animal sources that inhibits rotavirus infection and other pathogenic microorganisms, as well as being tolerant of gastric and bile acids. Also, this invention relates to a prophylactic and therapeutic composition comprising the same for contributing in many probiotic ways to the host's general health and preventing and treating diseases or conditions associated with rotavirus and other enteric pathogens.

1/2

## SEQUENCE LISTING

&lt;110&gt; Probionics Corp.

&lt;120&gt; NOVEL Lactobacillus reuteri USEFUL AS PROBIOTICS

&lt;130&gt; 2001-P-03

&lt;150&gt; KR

&lt;151&gt; 2001-03-07

&lt;160&gt; 1

&lt;170&gt; KopatentIn 1.71

&lt;210&gt; 1

&lt;211&gt; 1531

&lt;212&gt; DNA

&lt;213&gt; Lactobacillus reuteri

&lt;220&gt;

&lt;221&gt; rRNA

&lt;222&gt; (1)..(1531)

&lt;223&gt; 16S rDNA sequence of Lactobacillus reuteri Probio-16

&lt;400&gt; 1

gatgaacgcc ggcggtgtgc ctaatacatg caagtcgtac gcactggccc aactgattaa	60
tggtgcttgc acctgattga cgatggatca ccagtgagtg gcggacgggt gagtaacacg	120
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aacaaaagcc acatggcctt tggttgaaag atggccttgg ctatcactct gggatggacc	240
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2/2

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